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Gibberellin 2-Oxidases from Seedlings of Adzuki Bean (*Vigna angularis*) Show High Gibberellin-Binding Activity in the Presence of 2-Oxoglutarate and Co^{2+}

Seung-Hyun PARK,[1,*] Masatoshi NAKAJIMA,[1,*] Masayuki SAKANE,[1] Zheng-Jun XU,[2,**] Katsushi TOMIOKA,[1] and Isomaro YAMAGUCHI[1,†]

[1]*Department of Applied Biological Chemistry, University of Tokyo, Bunkyo, Tokyo 113-8657, Japan*
[2]*Rice Institute, Sichuan Agricultural University, Wenjiang District, Chengdu, 61130, China*

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Five full-length cDNA encoding gibberellin 2-oxidases, *VaGA2oxA1*, *VaGA2oxA2*, *VaGA2oxB1*, *VaGA2oxB2*, and *VaGA2oxB3*, were cloned from etiolated adzuki bean (*Vigna angularis* cv. Dainagon) seedlings, and their enzymatic characteristics were examined using recombinant enzymes fused with glutathione *S*-transferase (GST). Recombinant VaGA2oxA1 (rVaGA2oxA1) and rVaGA2oxA2 showed 2β-hydroxylation activity by converting GA_1, GA_4, GA_9, GA_{20}, GA_4-methyl ester, and 16,17-dihydro-GA_4 to the corresponding 2β-hydroxylated gibberellins, which were identified by GC/MS. rVaGA2oxB1, rVaGA2oxB2, and rVaGA2oxB3 showed similar activity by converting [$^3H_4$]-16,17-dihydro-GA_4 to a metabolite showing an R_f value of 16,17-dihydro-GA_{34}. RNA-blot analysis showed that *VaGA2oxA1* and *VaGA2oxA2* were the major ones expressed in etiolated hypocotyls. The addition of Co^{2+} instead of Fe^{2+} to the assay medium apparently reduced the enzymatic activity, but increased the binding of [$^3H_4$]-16,17-dihydro-GA_4 to rVaGA2oxA1, indicating the possibility that VaGA2oxs can be detected as gibberellin-binding proteins under certain conditions.

Key words: adzuki bean (*Vigna angularis*); gibberellin; gibberellin 2-oxidase; gibberellin-binding protein

Gibberellins (GAs) are a class of phytohormones that regulate diverse developmental processes in plant growth, including seed germination, stem elongation, leaf expansion, flowering, and fruit ripening, and mediate environmental stimuli.[1–3] The wide spectrum of GA action can be explained by a multiplicity in the regulation of its signal transduction and biosynthetic pathways that has been elucidated using many GA-related mutants.[2–4] The receptor(s) for GA, as well as tissue specific regulation of GA biosynthesis, might also contribute to the multiplicity of GA action through its tissue specific and temporal expressions, although they have not been identified.

Since the gibberellin-binding protein (GBP) in adzuki bean seedlings has shown high and specific affinity to biologically active GAs such as GA_1, GA_3, GA_4, and GA_7,[5] the catabolic enzymes for these GAs are candidates for proteins that show GA-binding activity. The genes that encode the enzymes catalyzing GA biosynthesis have been identified from many plant species mostly using GA-deficient mutants, and an almost complete cascade of the GA biosynthetic process has become clear.[6] The cDNA encoding GA 2-oxidase, which catalyzes the final step of GA biosynthesis,[7] was first cloned from runner bean by functional screening,[8] and clonings of its orthologs from many plant species have been achieved based on its sequence information. Meanwhile, the sequence information is available from a number of plant species such as *Arabidopsis thaliana*,[8] *Phaseolus coccineus*,[8] *Pisum sativum*,[9,10] *Oryza sativa*,[11] *Spinacia oleracea*,[12] *Cucurbita maxima*,[13] and *Lactuua sativa*.[14] The enzymatic characteristics of GA 2-oxidases have also been reported using their recombinant enzymes. Some GA 2-oxidases show substrate specificity to bioactive GAs carrying the 3β-hydroxy group,[9] but others show it to both bioactive GAs and their immediate biosynthetic precursors such as GA_9 and GA_{20},[6] or preferentially to the precursors.[8,14] But no information other than enzymatic characteristics has been reported for any of the GA 2-oxidases. If GA 2-oxidase that selectively catalyzes the oxidation of biologically active GAs exists in adzuki seedlings, it can be detected as an adzuki GBP under a condition in

* Both contributed equally to this research.
** Engaged in this research at the University of Tokyo as a postdoctoral fellow from the Bio-oriented Technology Research Advancement Institution (BRAIN).
† To whom correspondence should be addressed. Fax: +81-3-5841-8025; E-mail: aisomar@mail.ecc.u-tokyo.ac.jp
Abbreviations: DIG, digoxygenin; DW, distilled water; GAs, gibberellins; GA2ox, gibberellin 2-oxidase; GBP, gibberellin-binding protein; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; rVaGA2oxs, recombinant VaGA2oxs

which the release of GAs from GA-enzyme complex is interrupted. With a view to this, we cloned the cDNAs of GA 2-oxidase expressed in adzuki seedlings and examined their enzymatic characteristics using their recombinant proteins under different assay conditions. Here we report that VaGA2oxA1, a major GA 2-oxidase in adzuki hypocotyls, showed GA-binding activity under a specific assay condition.

Materials and Methods

Plant materials. Seeds of adzuki bean (*Vigna angularis* cv. Dainagon) were surface sterilized in NaClO (0.1%, 5 min), imbibed in the dark at 25 ± 2 °C overnight, seeded on an agar medium (0.7%), and grown at 25 ± 2 °C in the dark for 7 d. Arial parts were harvested and used for the experiments.

Chemical compounds and instruments. The gibberellins used in these experiments were prepared in our lab, and [1,2,16,17-$^3H_4$]-16,17-dihydro-GA_4 (4.55 TBq/mmol) was prepared by catalytic tritiation of GA_7-benzyl ester in Du Pont/NEN (Boston, MA) and purified by HPLC using a Senshu Pak $N(CH_3)_2$ column (8 mm i.d. × 150 mm, Senshu Scientific, Tokyo) and MeOH containing 0.05% AcOH as a developing solvent. GC/MS was carried out on a Hitachi M-4100 mass spectrometer connected with a HP-5890 series II gas chromatograph (Hitachi High-Technologies, Tokyo) under the following conditions: column, DB-5 (0.25 mm i.d. × 15 m, 0.25 μm thick; J&W Scientific, Foster City, CA); temperature program, 60 °C (2 min), 60 °C to 300 °C linear gradient at 15 °C/min; He flow, 1 ml/min; ion source temperature, 280 °C; ionization, EI (70 eV).

Cloning and sequencing of VaGA2oxs. The etiolated 7 d-old hypocotyls were soaked in a GA_4-solution (50 μM) for 8 h in the dark, and upper parts of 3 cm segments from the apical bud were used for preparation of total RNA. Poly(A^+) RNA was purified with a Dynabeads mRNA purification kit (Dynal, Oslo, Norway). cDNA was synthesized with a Marathon cDNA amplification kit (Clontech Laboratories, Palo Alto, CA) according to the manufacturer's protocols, and used as a template for PCR. Degenerated primers were designed on the basis of the well-conserved amino acid regions among GA 2-oxidases of dicotyledonous plants. Forward sense primers: F1, 5′-GGNTTYGGDGARCAYACWGAYCCWCA-3′; F2, 5′-GGNTTYGGNGARCAYACNGAYCCNCA-3′. Reverse antisense primers: R1, 5′-CTYYTRAAYCTYCCRTTDGTCATHACCT-3′; R2, 5′-CCDSMRAARTADATCATHGAHANCCT-3′; and R3, 5′-TAYTRSVACCAWGTRAAYTCHTYRTA-3′. PCR was performed using pairs of primers, F1 and R2, F2 and R1/R2/R3, and an adaptor primer and R1. The reaction was initiated by heating pairs of primers at 95 °C for 3 min and performed under the following conditions: 30 cycles of 95 °C for 30 s, 57.5 °C for 1 min, and 72 °C for 2 min. The reaction was completed by incubation at 72 °C for 5 min. PCR products were separated by agarose electrophoresis, and 0.2–0.3 kb fragments were recovered. The fragments were purified by a Geneclean (Bio 101, Vista, CA) according to the protocol, and ligated into a pGEM T-easy plasmid vector (Promega, Madison, WI) for transformation of JM109 *E. coli*. Five different cDNA fragments were obtained and designated *VaGA2oxA1*, *VaGA2oxA2*, *VaGA2oxB1*, *VaGA2oxB2*, and *VaGA2oxB3*. To obtain sequence information for full-length *VaGA2oxs*, 5′- and 3′-RACE were performed using the following primers designed from the sequences of the cDNA fragments: 5′-GTCTTAAGATCTAATAGCACATCAGGC-3′ (for 3′-RACE of *VaGA2oxA1*), 5′-TCTCCAACATTGATGAAGAAGGAGTTG-3′ and 5′-CTGCTTAGGTCTAACAACACTTCAGG-3′ (for 5′- and 3′-RACE of *VaGA2oxA2*), 5′-CATCGCCAACCATGACAAAGAAATTAG-3′ and 5′-GAATGTGGGTGGCCTTCAAATTTACAC-3′ (for 5′- and 3′-RACE of *VaGA2oxB1*), 5′-CACCGACGTTGACATAGAATGCAGAG-3′ and 5′-GTCGGAGGCCTCCAAATTTCTCTTCCA-3′ (for 5′- and 3′-RACE of *VaGA2oxB2*), and 5′-CATCACCAACCATAACGAAGAACTGAT-3′ and 5′-CAACGTGGACGGCCTTCAGATTTCCA-3′ (for 5′- and 3′-RACE of *VaGA2oxB3*). PCR was performed under the following conditions: initiation, 95 °C for 3 min, and 25 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 2 min. The reaction was completed by 7 min incubation at 72 °C. The coding regions for *VaGA2oxs* were obtained by PCR reactions with primers, which were designed according to the full-length cDNA sequences. The primers were 5′-GGAATTCCCATGGTTGTTCTGTCTCAGCCAAGCA-3′ and 5′-GGAATTCTTAATCAGTAGCAGATTTCTCGAAAGG-3′ (forward and reverse primers of *VaGA2oxA1*), 5′-CGGGATCCATGGCATCGTTGTGCCAAAACAACAAC-3′ and 5′-CGGGATCCTCTTGCATATTATGAAGCTGCAATTC-3′ (forward and reverse primers of *VaGA2oxA2*), 5′-GGAATTCCAATGGTTGTGCCTTCTCCAACATCC-3′ and 5′-GGAATTCAAVGTTGAAACTGAGATTGAGAGG-3′ (forward and reverse primers of *VaGA2oxB1*), 5′-GGAATTCAAATGGTGTTGGCTGCCCGAAACCCA-3′ and 5′-GGAATTCAGCGTTTGCAGTTTCTGAAAAGCTG-3′ (forward and reverse primers of *VaGA2oxB2*), and 5′-GGAATTCCAATGGTTGCTCCTTGTCCAACTTCC-3′ and 5′-GGAATTCTAAGTGTCAGGGAGAAGCGATGC-3′ (forward and reverse primers of *VaGA2oxB3*). *Bam*HI sites were added to both ends of each primer of *VaGA2oxA2*, and *Eco*RI sites were added at both ends of all other primers. Each cDNA fragment, which contained the complete coding region encoding the corresponding GA 2-oxidase, was ligated in the *Eco*RI site (*VaGA2oxA1*, *VaGA2oxB1*, *VaGA2oxB2*, and *VaGA2oxB3*) or the *Bam*HI site (*VaGA2oxA2*) of a pGEX-4T-2 vector (Amersham Biosciences, Piscataway, NJ) of the GST fusion system and cloned in JM

109 *E. coli*. The in-frame connection of each GA 2-oxidase-GST fusion gene was confirmed by sequencing according to the recommended sequencing primers for the fusion system.

RNA-Blot analysis. The dark-grown and etiolated 3 d-, 5 d-, and 7 d-old seedlings were used for RNA-blot analysis. Total RNA were isolated from the whole plant of 3 d-old seedlings and from the leaves, including shoot apexes (L) and hypocotyl segments (0–1 cm [UH], 1–4 cm [MH] from the first petiole junction, and lower part [LH]) of 5 d- and 7 d-old seedlings either untreated or treated 4 h previously with mixture of 0.1 mM GA_3 and GA_4. Each 10 μg of total RNA was separated on 1% (w/v) agarose gel containing 5% (v/v) HCHO, and transferred to a nylon membrane (Hybond-N^+, Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions. A probe (RNA) solution was prepared with a DIG RNA labeling kit (Boehringer Mannheim/Roche, Basel, Switzerland) and diluted (2,000 fold) in the hybridization buffer according to the manufacturer's instructions. After 3 h prehybridization at 65 °C, hybridization was performed overnight at 68 °C before detection.

Preparation of the recombinant proteins. The pGEX plasmid harboring the *VaGA2oxs* ORF-region was used to transform *E. coli* Rosetta™ (DE3)-competent cells (Novagen, Darmstadt, Germany) according to the manufacturer's protocol, before selecting the colonies on a Luria Bertani (LB) plate containing 1.5% (w/v) agar, 34 μg/ml chloramphenicol, and 100 μg/ml ampicillin. A single colony was selected and grown overnight at 37 °C. A 2.0 ml aliquot of the culture was inoculated into 100 ml of fresh LB broth containing 34 μg/ml chloramphenicol, 100 μg/ml ampicillin, and 2% (w/v) glucose, and shaken at 26.5 °C. When cell density (OD_{600}) reached 0.6, the fusion protein was induced by removing glucose by centrifugation (1,000 × *g*, 2 min) followed by the addition of isopropyl-β-D-thiogalactoside to 0.3 mM. After 5 h incubation, the cells were collected by centrifugation (10,000 × *g*, 10 min), and resuspended in 10 ml of DW containing 1 mg/ml lysozyme. After 10 min incubation, the suspension was frozen and kept at −80 °C overnight, thawed in water, chilled on ice, and sonicated. Soluble proteins were collected by centrifugation (10,000 × *g*, 10 min) and purified by affinity chromatography using a Glutathione Sepharose™ 4B (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's protocol, and stored at −80 °C.

Functional assay. Affinity purified recombinant proteins (*ca.* 1 μg) were dissolved into 50 μl of 100 mM Tris–HCl (pH 7.5) containing 1 mM 2-oxoglutarate, 1 mM $FeSO_4$, and 4 mM ascorbate. To this assay solution, 1 μg of substrate GA (GA_1, GA_4, GA_9, GA_{20}, and 16,17-dihydro-GA_4) was added.[15] After 2 h incubation at 30 °C, the enzymatic reaction was terminated by adding 5 μl of AcOH. The solution was applied to a C_{18} Sep-Pak™ cartridge (1 ml, Waters, Milford, MA), and eluted with 1.2 ml of DW and 2.5 ml of MeOH successively. The MeOH eluate was dried up *in vacuo*, and the concentrate was methylated with CH_2N_2 and trimethylsilylated in fresh *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA). An aliquot of the reaction mixture was subjected to GC/MS. Each product was identified by comparing its MS and Kovats' retention indices (KRI)[16] to those of the authentic specimen (Table 1).

GA-Binding assay. To 90 μl of the assay buffer of 50 mM Tris–HCl (pH 7.6) containing 0.1 M KCl and *ca.*

Table 1. Metabolism of Recombinant VaGA2oxAs

Substrate	Metabolite[a]	KRI	Characteristic ions (% Relative intensity)
GA_1	GA_8	2,843	594 (100), 448 (10), 379 (7), 238 (11), 207 (11), 143 (6), 73 (23)
GA_4	GA_{34}	2,719	506 (100), 459 (13), 416 (11), 387 (17), 372 (16), 356 (10), 313 (14), 288 (13), 272 (17), 241 (18), 223 (34), 217 (34)
GA_9	GA_{51}	2,596	418 (3), 403 (4), 386 (14), 371 (5), 343 (4), 328 (14), 315 (5), 299 (10), 296 (13), 284 (62), 268 (56), 241 (20), 225 (100), 209 (13), 183 (18)
GA_{20}	GA_{29}	2,723	506 (100), 491 (10), 447 (8), 389 (17), 375 (27), 303 (27), 277 (19), 235 (9), 207 (16), 167 (9)
16,17-dihydro-GA_4	16,17-dihydro-GA_{34}	2,767	508 (100), 461 (8), 420 (13), 374 (12), 345 (9), 315 (9), 291 (22), 263 (15), 235 (17), 181 (14), 157 (7), 129 (18), 103 (44)
GA_4-methyl ester	GA_{34}-methyl ester	2,722	506 (76), 479 (15), 459 (5), 418 (32), 371 (26), 345 (13), 311 (11), 289 (47), 261 (23), 233 (38), 181 (17), 159 (18), 129 (30), 91 (11), 73 (100)

Substrate GAs were incubated with recombinant VaGA2oxAs, and the metabolite GAs were identified by full-scan GC/MS and KRI.
[a]Identification of metabolites by GC/MS on the basis of KRI and full-scan mass spectra of Me ester TMSi ether derivatives.

0.66 pmol (110,000 dpm, 1.83 kBq) of [$^3H_4$]-16,17-dihydro-GA_4, *ca.* 0.2 μg of affinity purified protein in 10 μl of 50 mM Tris–HCl (pH 7.6) was added. After 1 h incubation at 30 °C, the mixture was subjected to a G-25 gel permeation column (NAP-5, Amersham Biosciences, Uppsala, Sweden) eluted with 50 mM Tris–HCl (pH 7.6) containing 0.1 M KCl. The radioactivity in the void fraction was measured. The specific binding was calculated by subtracting non-specific binding from total binding.[5]

Competition analysis. The assay for competition analysis was performed in the same manner as the functional analysis. GAs and their analogs of different amounts (0.03, 0.3, 3, 30, and 300 pmol) were incubated with *ca.* 0.3 pmol (50,000 dpm, 0.83 kBq) of [$^3H_4$]-16,17-dihydro-GA_4 at 30 °C for 2 h in 50 μl of a Tris–HCl buffer (100 mM, pH 7.5) containing 1 mM 2-oxoglutarate, 1 mM $FeSO_4$, and 4 mM ascorbate. The products were extracted twice with 50 μl of EtOAc, concentrated, and subjected to TLC developed in a solvent mixture of $CHCl_3$:EtOAc:AcOH (20:20:1, v/v). The radioactivity was visualized with an imaging plate (BAS-TR2040, Fuji Photo Film, Tokyo) and an imaging analyzer (FLA-3000G, Fuji Photo Film). The amounts of the metabolites and the substrate remaining were estimated as pixels by scanning the imaging spots of the metabolite and the substrate using software (Scion Image for Windows ver. 4.0.2).

For Dixon plot analysis, the radioactivity of the metabolite ([$^3H_4$]-16,17-dihydro-GA_{34}) and the substrate ([$^3H_4$]-16,17-dihydro-GA_4) was measured by online radiocounting (Ranoma-92, Raytest, Straubenhardt, Germany) with software (Gina ver. 4.6) after separation by HPLC on a Senshu Pak Pegasil ODS column (4.6 mm i.d. × 150 mm, Senshu Scientific) under the following conditions: flow rate, 1 ml/min; solvent program, 0–5 min; linear gradient, 10–27.5% CH_3CN in 0.5% AcOH, 5–10 min; 27.5% CH_3CN in 0.5% AcOH, 10–15 min; linear gradient, 27.5–80% CH_3CN in 0.5% AcOH, 15–20 min; 80% CH_3CN in 0.5% AcOH.

Results

Cloning of VaGA2oxs

cDNA template was prepared from total RNA from hypocotyls of GA_4-treated 7 d-old adzuki bean (*Vigna angularis* cv. Dainagon) seedlings. Because information about GA-biosynthetic enzymes of adzuki bean was not available on the database, degenerated primers were designed on the basis of well-conserved amino acid sequences among *GA 2-oxidase* genes of dicotyledonous plants. PCR was performed against the cDNA template using the degenerated primers, and some cDNA fragments were amplified. The combinations of PCR primers that gave cDNA fragments were as follows: A1, adaptor primer and R1; A2, F1 and R2; B1, F2 and R1; B2, F2 and R2; and B3, F2 and R3. After 5′- and 3′-RACEs using the forward and reverse primers described in "Materials and Methods", four clones were isolated and designated *VaGA2oxA2*, *VaGA2oxB1*, *VaGA2oxB2*, and *VaGA2oxB3*, and a clone, *VaGA2oxA1*, happened to be obtained by 3′-RACE alone, because its cDNA fragment had been obtained by PCR using an adaptor primer and the reverse antisense primer R1. They encoded proteins of 332 aa (VaGA2oxA1, M_r 36,900), 327 aa (VaGA2oxA2, M_r 37,000), 340 aa (VaGA2oxB1, M_r 38,000), 323 aa (VaGA2oxB2, M_r 35,800), and 355 aa (VaGA2oxB3, M_r 39,600) respectively. Figure 1 shows their sequences and homology, and Fig. 2 shows the phylogenetic relationships.

RNA-Blot analysis

We prepared DIG-labeled probes specific to each of five *VaGA2ox* clones for RNA-blot analysis, and examined their expression patterns using 3 d-, 5 d-, and 7 d-old etiolated seedlings. Five d and 7 d-old seedlings were examined after they were divided into four parts, leaves including shoot apexes (L), 0–1 cm (UH) and 1–4 cm (MH) hypocotyl segments from the petiole junction, and lower hypocotyls (LH), while the whole plant was used for the analysis of 3 d-old seedlings. Signals due to mRNAs of *VaGA2oxA1* and *VaGA2oxA2* were clearly detected in every part of all seedlings 3 d, 5 d, and 7 d old (Fig. 3). Their levels did not change much during the 3–7 d after imbibition. The signal due to *VaGA2oxB3* mRNA was detected in 3 d- and 5 d-old seedlings at very weak intensities and hardly observed in 7 d-old ones, while signals due to mRNAs of *VaGA2oxB1* and *VaGA2oxB2* were not detected in any part. No responsiveness to GA-treatment was observed in any clone at any stage examined.

Preparation of rVaGA2oxs and their enzymatic activities

Recombinant VaGA2oxs with a GST tag (rVaGA2oxs) were prepared by the *E. coli* expression system, affinity purified, and used for function assays. Both rVaGA2oxA1 and rVaGA2oxA2 converted GA_1, GA_4, GA_9, GA_{20}, and 16,17-dihydro-GA_4 to the corresponding 2β-hydroxylated GAs, which were identified by referring to their KRI values and fragmentation patterns in full-scan GC/MS after methylation and trimethylsilylation (Table 1). The enzymatic activity of rVaGA2oxB1, rVaGA2oxB2, and rVaGA2oxB3 was examined by monitoring the conversion of [$^3H_4$]-16,17-dihydro-GA_4 to a polar metabolite, because rVaGA2oxBs were obtained mostly in an insoluble form and were not available in sufficient quantities to perform the experiment for GC/MS analysis. All recombinants converted the substrate to a polar metabolite with the same R_f value of 16,17-dihydro-GA_{34} on TLC, suggesting that those recombinants also possessed 2β-hydroxylation activity. In 2 h incubation for the enzyme assay, no significant difference was observed between the enzymatic activities of rVaGA2oxA1 and rVaGA2oxA2

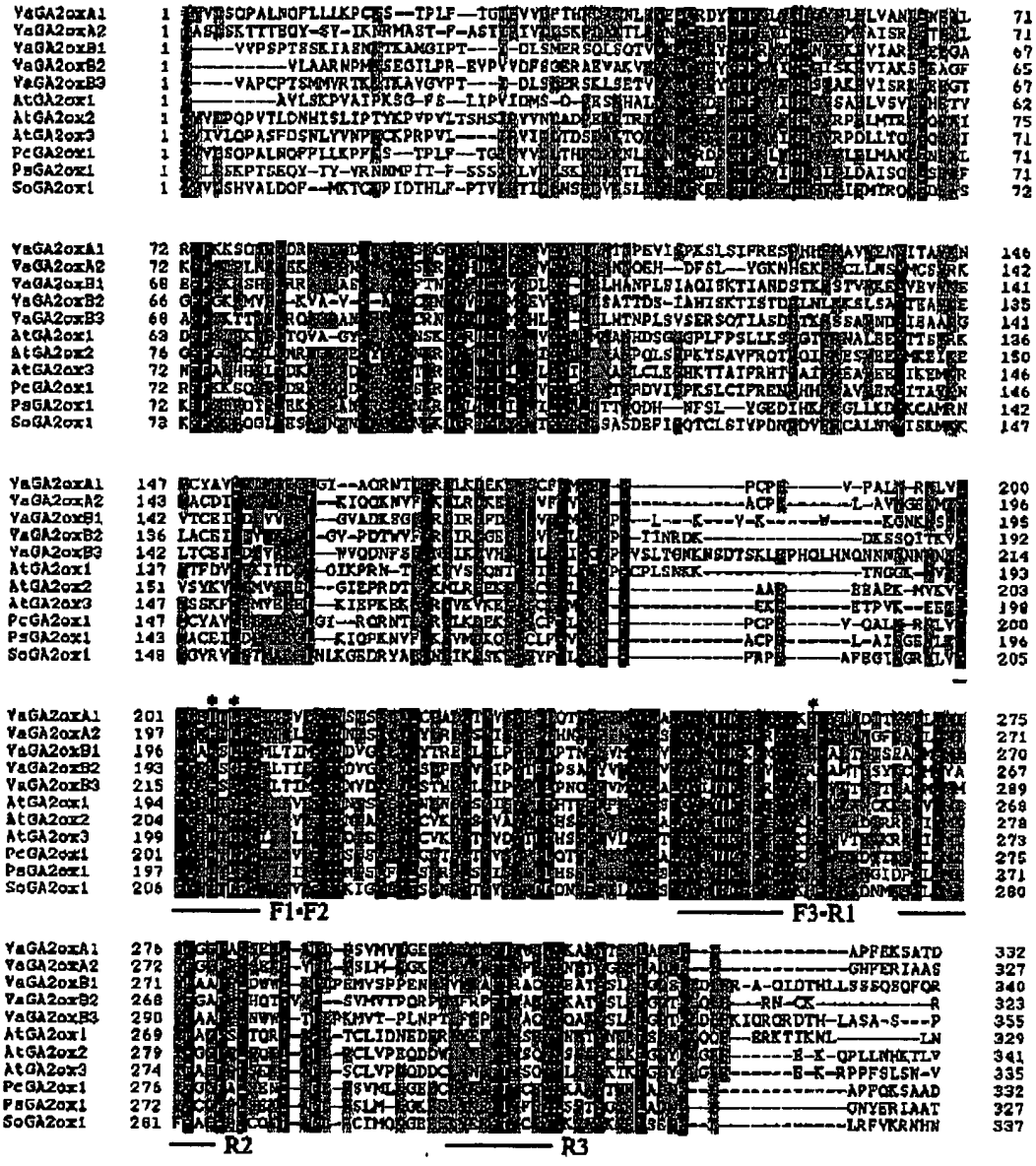

Fig. 1. Alignment of Deduced Amino Acid Sequences of VaGA2oxs and Other GA 2-Oxidases.

Dark shading indicates amino acid residues that are fully conserved in the GA 2-oxidases, and gray shading indicates similar residues. Three asterisks indicate amino acid residues presumed to bind Fe^{2+} at the active site. The regions corresponding to the primer sequences used for PCR are underlined. The sequence data for VaGA2oxs will appear in the DDBJ/EMBL/GenBank nucleotide databases; accession nos. AB181372 (VaGA2oxA1), AB181373 (VaGA2oxA2), AB181374 (VaGA2oxB1), AB181375 (VaGA2oxB2), and AB181376 (VaGA2oxB3). AtGA2ox1 (AJ132435), AtGA2ox2 (AJ132436), and AtGA2ox3 (AJ132437) are from *A. thaliana*;[9] PcGA2ox1 (AT132438) from *P. coccineus*;[8] PsGA2ox1 (AF100954) from *P. sativum*;[9] SoGA2ox1 (AF506281) from *S. oleracea*.[12] The alignment was generated using GENETYX (version 9.0).

(data not shown). In all assays, no GST controls showed any enzymatic activity.

The effect of Fe^{2+} and Co^{2+} on the enzymatic activity of rVaGA2oxA1 was examined, because *VaGA2oxA1* and *VaGA2oxA2* were the major ones expressed in adzuki hypocotyls that were used for purification of adzuki GBP,[5] and because no clear difference was observed between their enzymatic activities. Without 2-oxogultarate, no enzymatic activity at all was observed. Fe^{2+} was also required for the activity, while Co^{2+} did not successfully replace Fe^{2+} for the enzymatic activity, but increased the binding of the tracer to rVaGA2oxA1. The radioactivity of the tracer retained in a protein fraction was slightly higher in incubation with Fe^{2+} for the enzyme assay (1,209 ± 105 dpm) than in incubation without Fe^{2+} (521 ± 6 dpm), while it was much higher in incubation with Co^{2+} (3,409 ± 57 dpm) in place of Fe^{2+}. Contrary to the effect of Co^{2+} on rVaGA2oxA1,

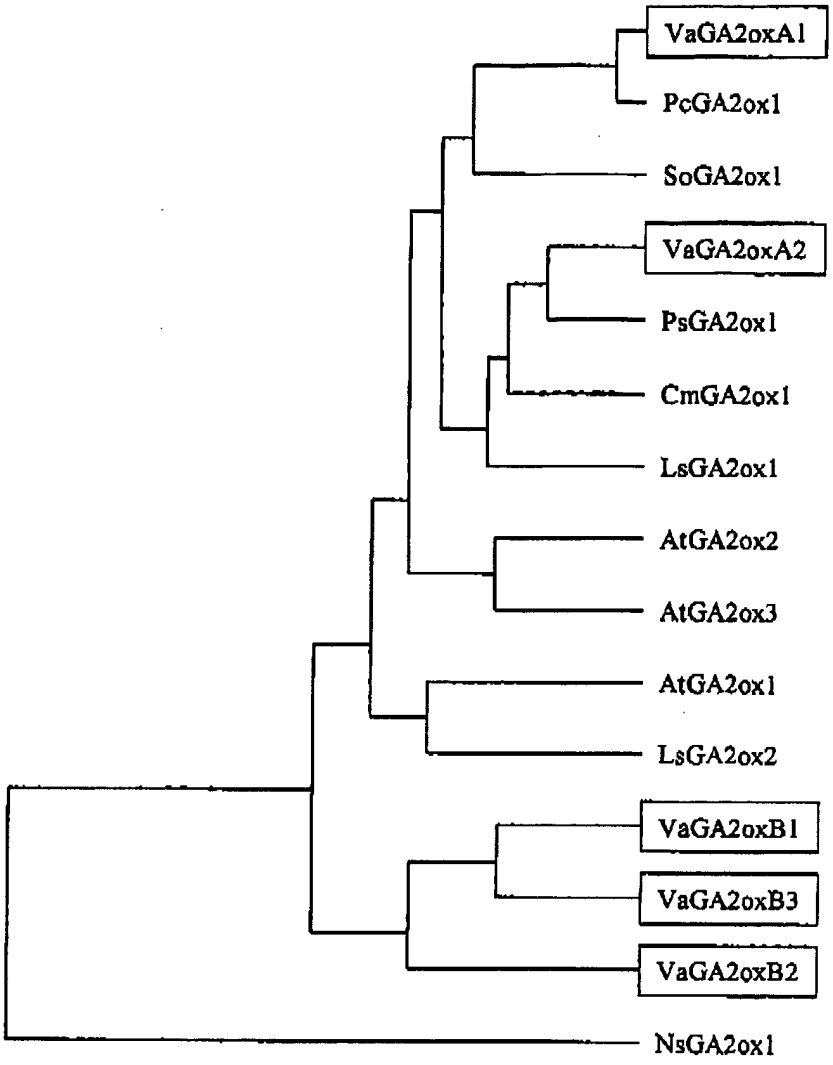

Fig. 2. Phylogenetic Relationships among GA 2-Oxidases.
Pc, *P. coccineus*; So, *S. oleracea*; Ps, *P. sativum*; Cm, *C. maxima*; Ls, *L. sativa*; At, *A. thaliana*; Ns, *N. sylvestryis*. The tree was generated using the UPGMA program in GENETYX (version 9.0).

Co^{2+} did not increase the amounts of the tracer bound to rVaGA2oxA2 (135 ± 16 dpm with Fe^{2+}, and 131 ± 30 dpm with Co^{2+}, Fig. 4). although it apparently inhibited the enzymatic activity.

The substrate selectivity of rVaGA2oxs was examined by monitoring the inhibition of the conversion of [$^3H_4$]-16,17-dihydro-GA_4 to its metabolite that was expected to be [$^3H_4$]-16,17-dihydro-GA_{34}. The addition of GAs into the assay medium for enzyme assay inhibited the conversion depending on both the species of GAs added and the recombinant enzymes. The levels of inhibition by GAs on the metabolism of the radioactive substrate by rVaGA2oxA1 and rVaGA2oxB2 are summarized in Fig. 5. No conversion of the tracer to a polar metabolite was detected when 30 pmol of GA_4 was added to the assay mixture, but it was still observed when 300 pmol of GA_9 or 3-*epi*-GA_4 were added. A similar tendency was observed in the competition assay of rVaGA2oxA2.

GA_3 and GA_4-methyl ester unexpectedly inhibited the oxidation of [$^3H_4$]-16,17-dihydro-GA_4 in each assay of all recombinants, although GA_3 cannot be hydroxylated at C-2, and GA_4-methyl ester is not native in plants. We clearly observed that rVaGA2oxA1 converted GA_4-methyl ester to GA_{34}-methyl ester, which was identified by GC/MS as its TMSi derivative (Table 1). We examined whether GA_3 inhibited the oxidation of the tracer by competing for the binding site in rVaGA2oxA1 or by allosteric effect. The competition between GA_3 and the radio tracer was evaluated by adding increasing amounts of GA_3 to the assay solutions containing the tracer at concentrations of 0.3 pM and 0.6 pM. The Dixon plots are shown in Fig. 6.[17]

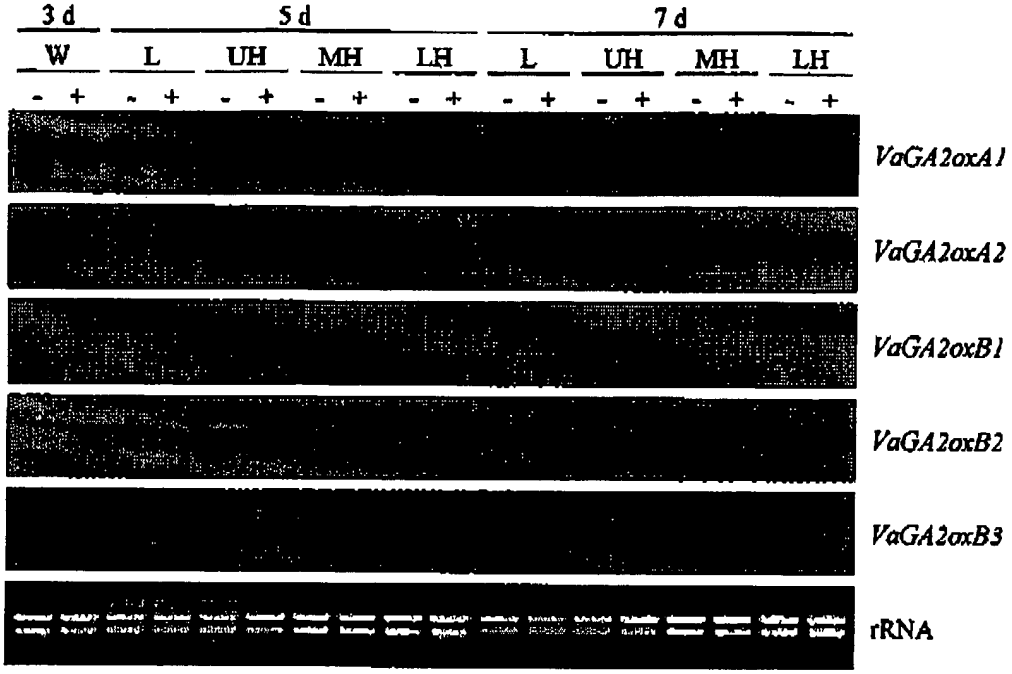

Fig. 3. RNA-Blot Analysis of *VaGA2oxs* Transcripts Levels in the Segments of Adzuki Bean Seedlings.

The dark-grown and etiolated 3 d-, 5 d-, and 7 d-old seedlings of adzuki bean were used for RNA-blot analysis. Total RNA (10 μg) was extracted from whole plant of 3 d-old seedlings and from the leaves and hypocotyl segments of 5 d- and 7 d-old seedlings. W, whole seedlings; L, leaves including apical buds; UH, 0–1 cm hypocotyls segments from petiole junction; MH, 1–4 cm hypocotyls segments from petiole junction; LH, lower hypocotyls; −, untreated seedlings; +, seedlings treated with a mixture of 0.1 mM GA_3 and GA_4 for 4 h before harvesting.

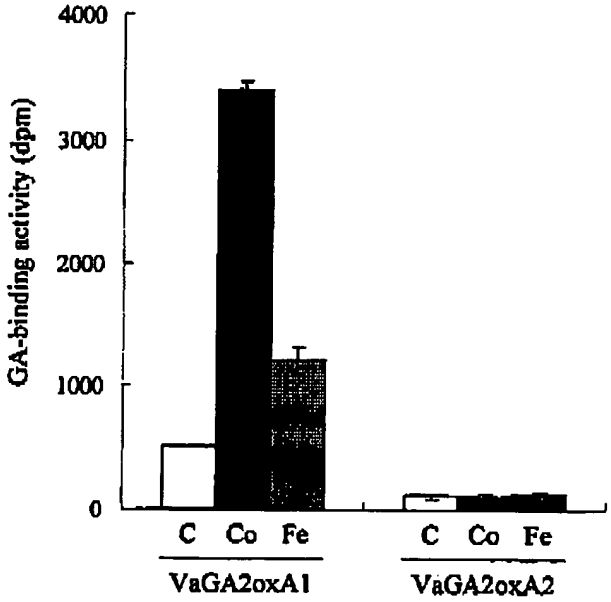

Fig. 4. GA-Binding Activity of rVaGA2oxA1 and rVaGA2oxA2.

Affinity purified rVaGA2oxA1 and rVaGA2oxA2 were subjected to GA-binding assay using 0.66 pmol of [$^3H_4$]-16,17-dihydro-GA_4 (110,000 dpm, 1.83 kBq) and binding activity was determined by subtracting unspecific binding from total binding. The values represent the mean of three independent experiments and SD. C, Control (Tris buffer); Co, 1 mM Co^{2+} in Tris buffer; Fe, 1 mM Fe^{2+} in Tris buffer.

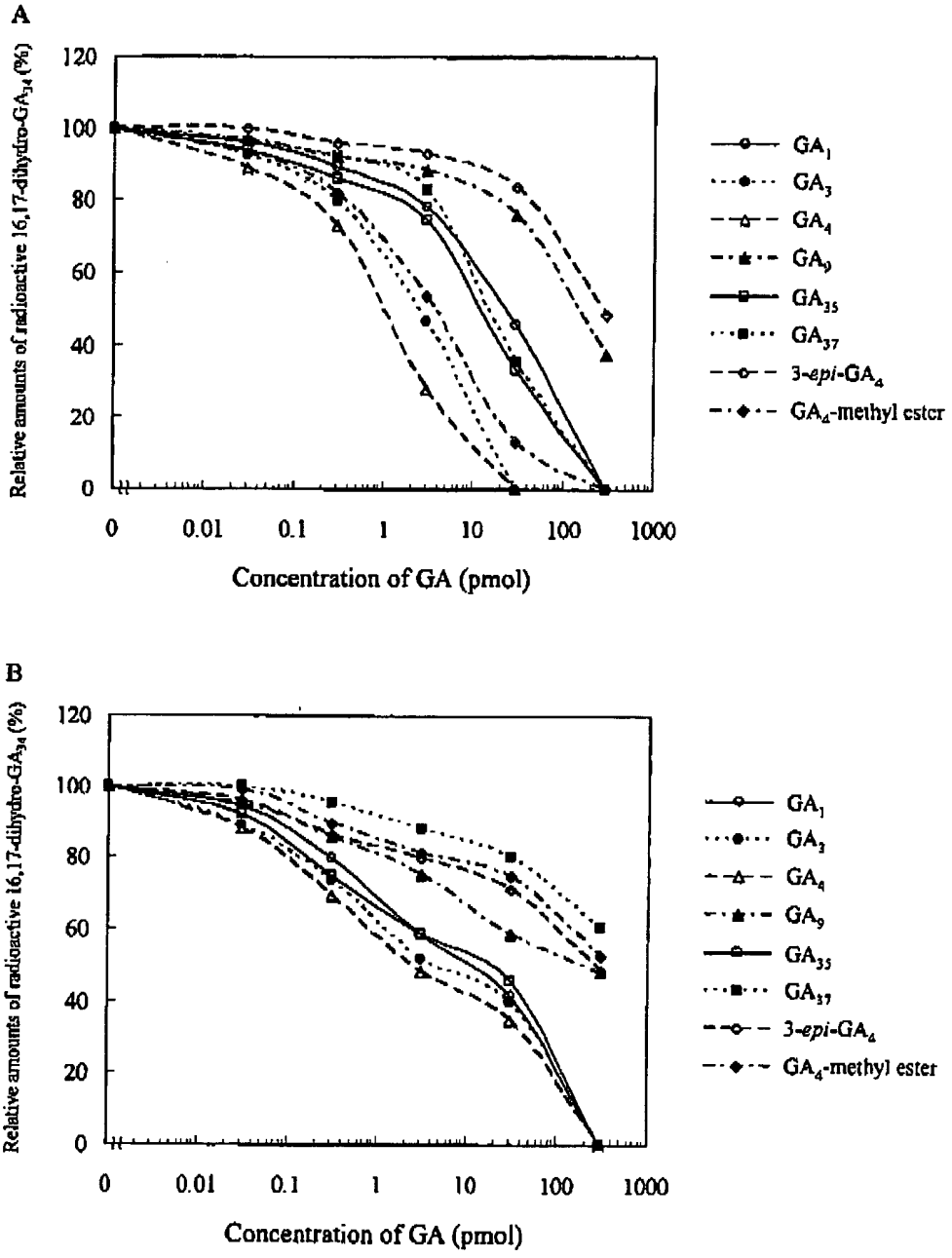

Fig. 5. Inhibition Effect of Various GAs on Metabolism of [$^3H_4$]-16,17-Dihydro-GA_4 by rVaGA2oxs.

Increasing amounts of GAs were added to the reaction mixture of the recombinant enzymes and a tracer ([$^3H_4$]-16,17-dihydro-GA_4, 50,000 dpm (0.83 kBq), 0.3 pmol). The amount of the metabolite was determined as the pixel values of the spot, and normalized against the amount of the metabolite produced without inhibiting GAs. The Y-axis expresses the normalized values. The values represent the mean of three independent experiments. In the assay of rVaGA2oxA1, SD was the largest at 30 pmol of GA_1 (4.2), and the others were smaller than 3.8. In the assay of rVuGA2oxB2. SD was the largest at 3 pmol of GA_1 (4.5), and others were smaller than 3.7. A, VaGA2oxA1; B, VaGA2oxB2.

Discussion

Clonings and sequences

Degenerated primers for PCR were constructed referring to the well-conserved sequences of known *GA 2-oxidase* genes from various plant species. We prepared PCR products with the constructed primers, and isolated five distinct cDNAs encoding GA 2-oxidases, *VaGA2oxA1*, *VaGA2oxA2*, *VaGA2oxB1*, *VaGA2oxB2*, and *VaGA2oxB3*, which shows that the enzymes in *V. angularis* are encoded by a small gene family, as in *A. thaliana*.[8] A homology search with the deduced amino acid sequences showed strong homology of these clones to GA 2-oxidases from *A. thaliana*,[8]

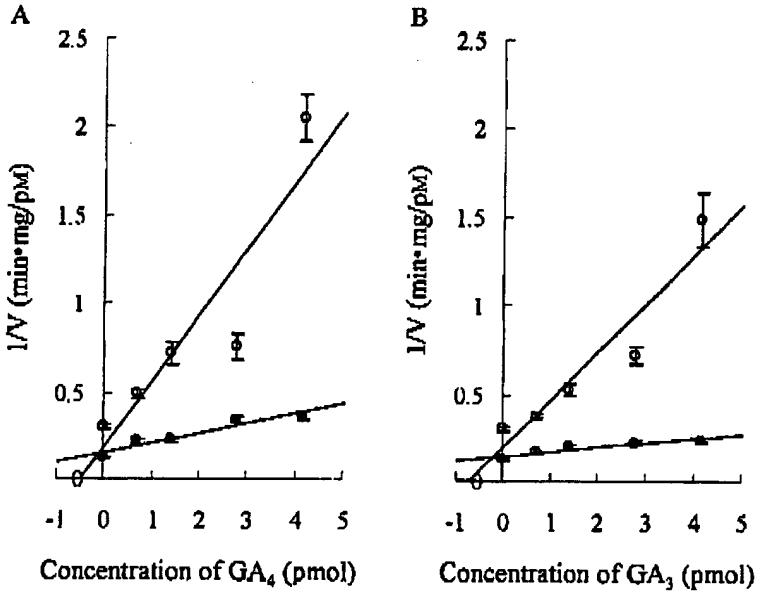

Fig. 6. Inhibition Kinetics of GA_4 (left) and GA_3 (right).
The enzymatic activity of rVaGA2oxA1 was measured in an assay mixture of 0.3 pM (○, 50,000 dpm, 0.83 kBq) and 0.6 pM (●, 100,000 dpm, 1.66 kBq) of [$^3H_4$]-16,17-dihydro-GA_4, and GA_4 (A) or GA_3 (B) at the indicated concentrations. The amount of rVaGA2oxA1 was *ca.* 62.5 ng in each assay. The values represent the means of three independent experiments and SD.

P. coccineus,[8] *P. sativum*,[10] and *S. oleracea*[12] (Fig. 1). Amino acid alignment indicated high conservation across these species that included Fe^{2+}- and 2-oxoglutarate-binding domains. Judging by the analogy with cephalosporin synthase,[18] the domain including His-204, Asp-206, and His-261 (the numbers refer to the VaGA2oxA1 sequence) is expected to bind Fe^{2+} at the active site. The phylogenetic relationship showed that VaGA2oxA1 and VaGA2oxA2 share relatively high amino acid identity with GA 2-oxidases from dicotyledonous plants, while VaGA2oxB1, VaGA2oxB2, and VaGA2oxB3 share significantly lower identity with them (Fig. 2). The alignment of the sequences shows some divergence among the VaGA2oxs in terms of gaps and residue identity.

RNA-Blot analysis

Among the five *VaGA2oxs*, *VaGA2oxA1* and *VaGA2oxA2* showed clear signals in leaves and hypocotyls in RNA-blot analysis (Fig. 3). Their signals were observed in all samples examined (3 d- to 7 d-old seedlings). No difference was observed between the transcription levels of the genes in the GA-treated and non-GA-treated segments, suggesting that the genes are not GA-responsive or that their expressions were nearly saturated. Among the three *VaGA2oxBs*, only the signal due to *VaGA2oxB3* was observed, with weak intensities, in 3 d- and 5 d-old seedlings. *VaGA2oxA1* showed the highest intensity among all *VaGA2oxs*, and *VaGA2oxA2* followed. These observations suggest that *VaGA2oxAs* were predominantly expressed and responsible for the metabolism of GAs in the hypocotyls.

Enzymatic activities and substrate specificities

Affinity purified GST-fused recombinant enzymes were used for enzymatic and competitive assays. Both rVaGA2oxA1 and rVaGA2oxA2 showed broad substrate specificity by converting GA_1, GA_4, GA_9, GA_{20}, 16,17-dihydro-GA_4, and GA_4-methyl ester to their corresponding 2β-hydroxylated products (Table 1). In the competition assay between radioactive [$^3H_4$]-16,17-dihydro-GA_4 and competitor GAs on rVaGA2oxA1 and rVaGA2oxB2, GA_4 showed the strongest inhibition on the 2β-hydroxylation of the radio tracer, and GA_3, GA_{35}, and GA_1 followed, suggesting that both GA 2-oxidases prefer to catalyze the 2β-hydroxylation of biologically active GAs. Clear differences were observed, however, between the effects of GA_{37} and GA_4-methyl ester on rVaGA2oxA1 and rVaGA2oxB2. GA_{37} showed moderate inhibition on the former and the weakest inhibition on the latter among the tested GAs, while the inhibition effect of GA_4-methyl ester was as strong as GA_3 on rVaGA2oxA1 but very weak on rVaGA2oxB2 (Fig. 5). This shows that these two GA 2-oxidases have different substrate specificity, reflecting their phylogenetic differences.

The kinetics of the inhibition by GA_3 were examined to clarify whether GA_3 inhibits the enzymatic activity competitively or allosterically, and the results are summarized in Fig. 6. The Dixon plots show that GA_3 as well as GA_4 competes for the same active site with the radio tracer by intersecting in the second part of the quadrant.[17]

The characteristic properties observed in PsGA2ox1, which preferentially catalyzes the 2β-hydroxylation of

3β-hydroxylated GAs such as GA_1 but its precursor, GA_{20},[9] very slightly, were hardly observed in any of the rVaGA2oxs, although a tendency of rVaGA2oxA1 and rVaGA2oxA2 to prefer GA_4 to GA_9 was noticed (Fig. 5A).

rVaGA2oxA1 and rVaGA2oxA2 showed similar characteristics in enzymatic activity, reflecting their similarity of 61% at deduced amino acid levels. They showed quite different properties, however, in binding to 16,17-dihydro-GA_4 in the presence of Co^{2+} in place of Fe^{2+} in the assay mixture. Further investigation is required to explain the difference between rVaGA2oxA1 and rVaGA2oxA2.

The above observations show that a comparison of the characteristics, including chromatographic behaviors and substrate specificity, of VaGA2oxA1 and adzuki GBP should afford useful information on the identification of adzuki GBP.

Acknowledgments

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